

New generation of bactericidal silver nanoparticles against different antibiotic resistant *Escherichia coli* strains

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Abstract Biosynthesis of silver nanoparticles (AgNPs) with ecofriendly plant extracts contains different phytochemicals act as both reducing and capping agents forming stable and shape-controlled AgNPs. The present study deals with the synthesis of AgNPs using the aqueous leaf extract of *Coleus aromaticus* and to evaluate the bactericidal activity against three antibiotic resistant *Escherichia coli* strains. The AgNPs are characterized using UV–Visible Spectroscopy, Scanning Electron Microscope coupled with X-ray Energy Dispersive Spectroscopy, Fourier transform-infrared spectroscopy, and Atomic force microscope. The synthesized AgNPs were crystalline in nature, spherical in shape with an average of 48 ± 5 nm in size, and the stability of AgNPs is due to its high negative Zeta potential -65.7 mV. The AgNPs have outstanding bactericidal activity against three antibiotic resistant *E. coli* strains. This is the first report of antibacterial activity against antibiotic resistant strains using AgNPs by green route.

Keywords Green route · AgNPs · Antibiotic resistant *E. coli* · Bactericidal activity

Introduction

Integration of biosynthesis and nanotechnology has opened new possibilities, in particular for biomedical applications, and the synthesis of noble metal nanoparticles, for various applications such as catalysis, electronics, optics, environmental and biotechnology (Hussain et al. 2003; Yuan 2005; Masciangioli and Zhang 2003; Albrecht et al. 2006). Silver, gold, and copper have been used regularly for the synthesis of stable dispersions of nanoparticles, which are very efficient in areas such as biological labeling, photonics, and surface-enhanced Raman scattering (SERS) detection (Smith et al. 2006; Kearns et al. 2006). Silver nanoparticles are used in the field of biosensing, imaging, drug delivery, nanodevice fabrication, and medicine (Nair and Laurencin 2007; Lee and El-Sayed 2006; Jain et al. 2008). The biosynthesis by employing plant extracts has drawn attention as a simple, rapid, stable, inexpensive, and viable alternative to chemical procedures and physical methods. Recently, plant sources have been widely used for the synthesis of silver nanoparticles by using, *Andropogon paniculata* (Kotakadi et al. 2014), *Catharanthus roseus* (Kotakadi et al. 2013), *Murraya koenigii* leaf (Philip et al. 2011), *Mangosteen* leaf (Veerasamy et al. 2011), *Mangifera indica* leaf (Philip 2011), Tansy fruit (Dubey et al. 2010), *Jatropha curcas* (Bar et al. 2009), *Cinnamomum zeylanicum* leaf (Smitha et al. 2009), *Camellia sinensis* (Nestor et al. 2008), Aloe vera (Chandran et al. 2006), and *Medicago sativa* seed extract (Lukman et al. 2011) have been used for the synthesis of metal nanoparticles of different sizes.

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It is well known that the silver ions and silver-based compounds are highly toxic in nature to microorganisms; this toxicity depends upon the preparation of the nano-sized particles which are specific in size and shape, and exhibits different physical and chemical properties of great interest (Joguet et al. 2002). Past studies shown that highly concentrated and nonhazardous nanosized silver particles can easily be prepared in a cost-effective manner (Sondi et al. 2003), Sondi and others also worked using synthesized AgNPs against different *Escherichia coli* bacteria showed good bactericidal activity (Sondi and Salopek-Sondi 2004). Audra I. Lukman et al. also worked on the antimicrobial and antibacterial properties of Ag nanoparticles against *Salmonella*, *Shigella*, and *Proteus* (Lukman et al. 2011). Recent studies have been focused on some of the physicochemical properties of AgNPs, and these physical or chemical properties are responsible for the effect on microorganisms. It also depends on concentration of AgNPs' involved in inactivation of *E. coli* (Dror-Ehre et al. 2009), and the antibiotic ability of AgNPs against *E. coli*, *Staphylococcus aureus*, *B. subtilis*, and *K. mobilis* was enhanced with increasing the silver content (Zhang et al. 2008). Flores and coworkers also studied the biocide activity of the AgNP-modified titanium substrates on *Pseudomonas aeruginosa* a gram-negative bacterium. *P. aeruginosa* is an opportunistic microorganism that can cause severe, life-threatening infections (Flores et al. 2010). And presently, the silver nanoparticles are also used as Diagnostic Antimicrobial Nanoparticles (DANs) The minimum inhibitory concentration of DANs suggests that they are slightly more effective against *S. aureus* than *E. coli* at 12 and 24 ppm, respectively (Mark Hoppens et al. 2014), whereas Ruparelia et al. determined the average MIC of SNPs against four different strains of *E. coli* and three different strains of *S. aureus* as 120 and 250 ppm, respectively (Ruparelia et al. 2008). Though there are several reports regarding antimicrobial activity of AgNPs, which can be prepared in a simple and cost-effective manner, suitable for the formulation of new types of bactericidal materials (Kotakadi et al. 2013, 2014), there are no reports regarding the studies of AgNPs on antibiotic resistant bacteria so far. So the present study deals the synthesis of AgNPs using leaf extract of *Coleus aromaticus* and to study bactericidal activity of these nontoxic nanomaterials.

Coleus aromaticus Benth is commonly known as Indian country borage and 'Kapparillaku' in Telugu (Kumar et al. 2007). *Coleus aromaticus* is used for seasoning meat dishes and food products, and decoction of its leaves is administered in cases of chronic cough and asthma. Other important medicinal properties viz, antispasmodic, headache, epilepsy, dyspepsia, diarrhea, nervous tension, rheumatism, whooping cough, and bronchitis (Warrier et al. 1995).

Coleus aromaticus is a good source of nutritious food supplement as it contains important phytochemicals like flavones salvigenin, quercetin, chrysoeriol, luteolin, apigenin, the flavanone eriodictol and the flavanol taxifolin, triterpenic acids, oleanolic acid, 2,3-dihydroxyoleanolic acid, ursolic acid, pomolic acid, tormentic acid, etc., (Sahaykhare et al. 2011).

Materials and Methods

Synthesis of Silver Nanoparticles

The Kapparillaku leaves were collected from the plants in and around Tirupati (Supplementary Fig. S1). The leaves were shade dried at room temperature and then made into fine powder with the help of electric blender. The plant leaf extract was prepared, by taking 3 g of fine powder leaves in a 250-mL Erlenmeyer flask with 150 mL of distilled water, and the mixture was boiled for 2–3 min and filtered through sterile muslin cloth and through whatmann filter paper. The filtrate was used as source of extract for the preparation of silver nanoparticles. To 5 ml diluted leaf extract, 10 ml of 0.001 (M) AgNO₃ was added and the sample was left at room temperature, until the color of solution changed from colorless to light brown and subsequently dark brown. In the present investigation, the production of AgNPs was carried out with leaf extract without any man-made chemicals.

Characterization

The bio-reduction of pure Ag⁺ ions done with the leaf extract of Kapparillaku was monitored periodically by sampling of the 1 mL aliquots, and the optical absorbance was recorded by UV–Vis Spectrophotometer (Nanodrop 8000 series Thermofisher) in 200–800 nm wavelength range. Scanning Electron Microscopy (SEM) and EDX was performed by Oxford Inca Penta FeTX3 EDS instrument attached to Carl Zeiss EVO MA 15 Scanning Electron Microscope (200 kV) machine with a line resolution 2.32 (in Å). The SEM images were taken by coating a drop of AgNPs on an aluminum foil. Energy dispersive absorption spectroscopy of AgNPs was carried out by the SEM equipment, as mentioned above. The FT-IR was carried using Brucker Tensor 27; Particle size and zeta potential measurement experiments were carried out using a Nanopartica SZ-100 (HORIBA). Morphological studies of AgNPs were also done by AFM. A drop of synthesized AgNPs suspension was placed on thin aluminum sheet and dried at room temperature, prior to analysis of the sample. (AFM-Solver Next, NT-MDT, Russia).

Antimicrobial Activity

The antimicrobial activity of silver nanoparticles was evaluated against three different strains of Gram-negative *E. coli* as follows: *E. coli* Strain-I (Donor) rifampin resistant, *E. coli* Strain-II (Recipient) streptomycin resistant, and *E. coli* AB1157 (Mutant) streptomycin resistant by disk method. Bacterial cultures were prepared freshly by transferring one to three colonies into a tube containing 20 ml nutrient broth (Himedia, gm/L) and grown overnight at 37 °C. Three replicates of respective microorganisms were prepared by spreading 100 µl of culture on the nutrient agar plate with the help of spreader. Disks were prepared using Whatmann No. 1 filter paper. The disks were placed on agar plates, and sample of synthesized silver nanoparticles 30 µl (30mcg) was added on the disk with the help of micropipette. The plates were incubated at 37 °C overnight. Amoxyclav (Himedia SD063, 30 mcg) disk was used as reference drug.

Results and Discussion

The water soluble ingredients present in the extract are responsible for the reduction of metal ions to form stable AgNPs. Leaves of *Coleus aromaticus* contain essential oil rich in carvacrol, thymol, eugenol, chavicol, and ethyl salicylate. Oil extracted through steam distillation and solid phase micro extractions has very good antimicrobial activity (Perumal et al. 2004).

UV–Visible Spectral Analysis

UV–Vis spectroscopy is an important tool to observe the formation of metal nanoparticles. In the present study, colorless leaf extract is known to contain several water soluble phyto-constituents which have the potential to reduce the silver ions to AgNPs by changing light yellow to dark brown colors (Fig. 1a, b). The surface plasmon resonance spectrum of synthesized AgNPs was observed at 423 nm after 10 min (Fig. 2). The particles in SPR region of around 410–450 nm can be attributed to spherical nanoparticles (Zaheer and Rafiuddin 2012).

SEM and EDX Analysis of AgNPs

The morphology of the synthesized AgNPs was determined by SEM, and it shows that the AgNPs are more uniform and spherical in shape (Fig. 3). The EDX data reveals that very strong signal to silver and weak signals to other elements like oxygen, Mn, and Iron which indicates the reduction of the silver ions to elemental silver, and the strong signal of aluminium is due to aluminium sheet being used as base for

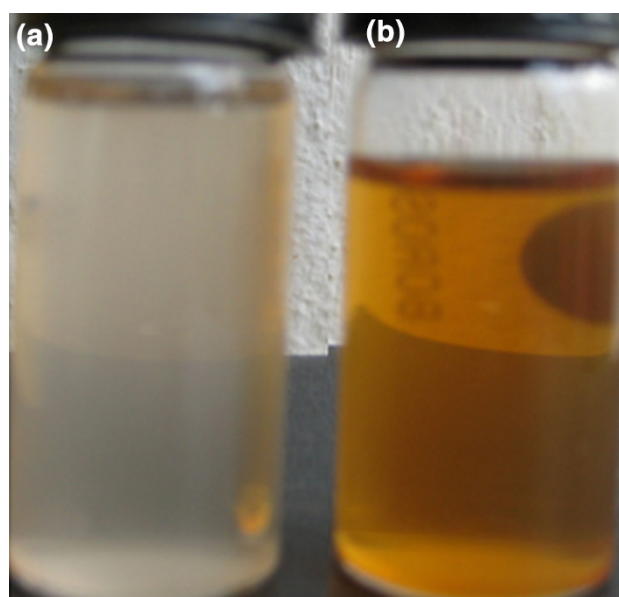


Fig. 1 **a** Aqueous extract of Kapparillaku and **b** AgNPs solution after addition of extract to AgNO₃ solution

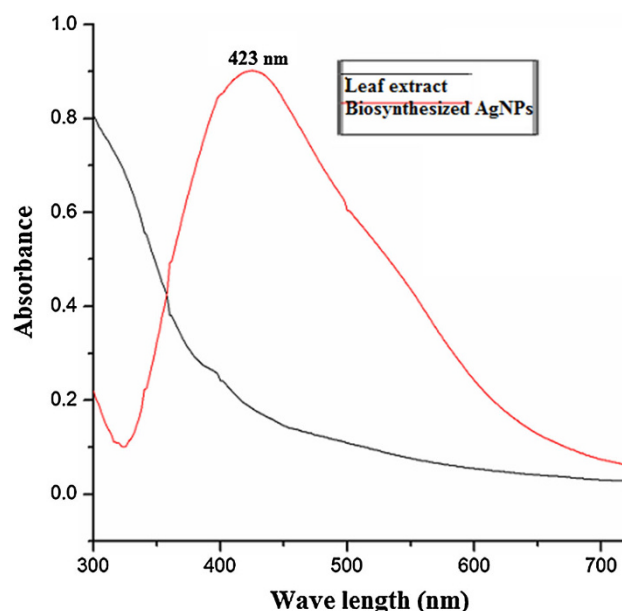


Fig. 2 UV–VIS absorption spectra of AgNPs synthesized from Kapparillaku leaves extract with 1×10^{-3} M silver nitrate

coating the silver nanoparticle sample for the study (Fig. 4). *Coleus aromaticus* plant leaves contain proteins, vitamins (ascorbic acid, thiamine), minerals (calcium, phosphorus, potassium, sodium, and magnesium), and trace metals (iron, zinc, copper, and chromium) (Sahaykhare et al. 2011).

IR Spectral Analysis

The IR spectrum of the silver nanoparticles is shown in (Supplementary Fig. S2), and the spectrum reveals that

hydroxyl group and stretch of alkane H (O–H, 3332.25; C–H, 2896.91, 2834.33) and carbonyl group and stretch of alkenes groups (C=C, 2096.66; C=O, 1637.26 cm^{-1}) are involved in the reduction of Ag^+ to Ag. Therefore, it may be conclude that the water soluble essential oils are responsible for capping and efficient stabilization (Sahaykhare et al. 2011).

Particle Size Determination

The particle size of the AgNPs obtained is detected by intensity and laser diffraction, which are poly-dispersed mixture solution. The size of synthesized AgNPs is ranging from 48 to 125 nm, and the average diameter of the AgNPs was found to be 53.2 nm (Fig. 5).

Zeta Potential of AgNPs

The negative value of Zeta potential confirms the repulsion among the particles and thereby increases in stability of the formulation. The zeta potential was found to be -69.5 mV (Fig. 6). The electrostatic repulsive forces between the nanoparticles when they are negatively charged possibly protect them from forming an association. This prevents the particles from agglomeration in the medium leading to long-term stability (Suresh et al. 2011).

AFM Analysis

The morphology of the synthesized AgNPs was spherical in shape, and the deposited AgNPs form aggregates on the

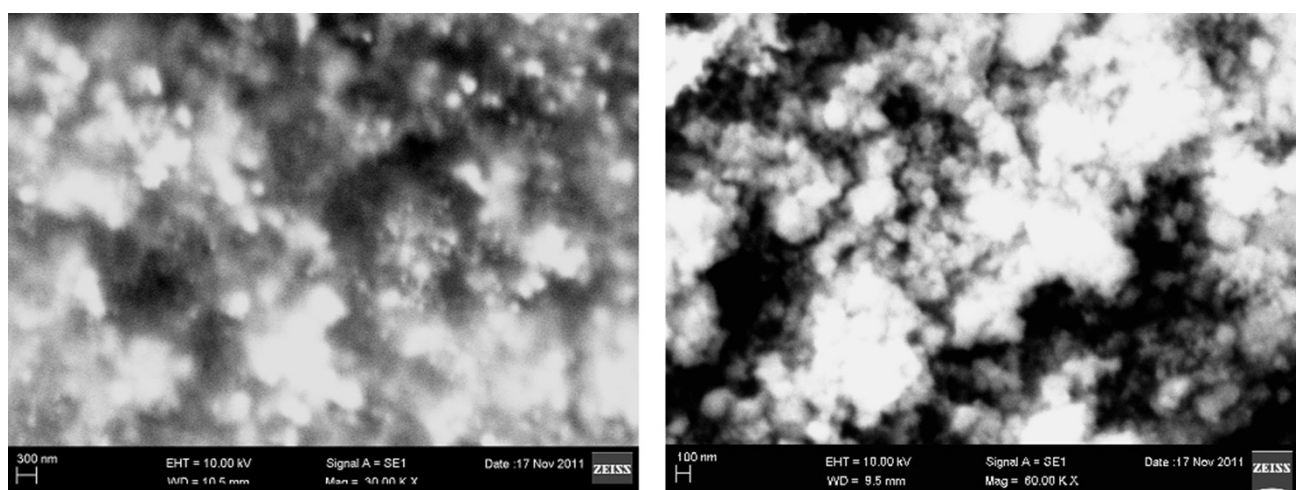


Fig. 3 SEM micrograph of synthesized AgNPs

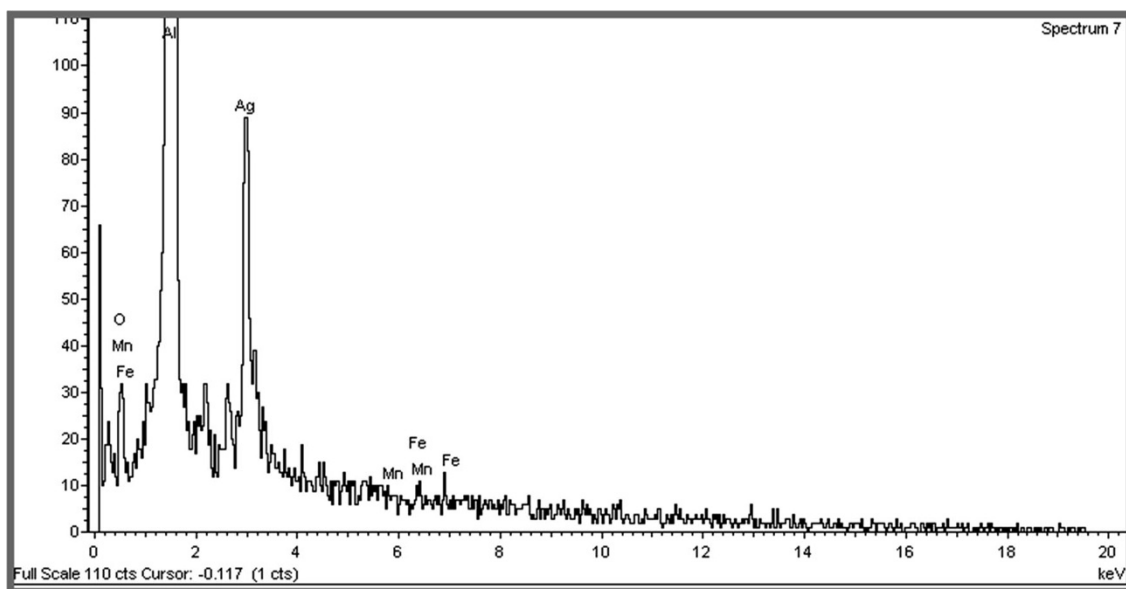


Fig. 4 EDX spectrum of synthesized silver nanoparticles

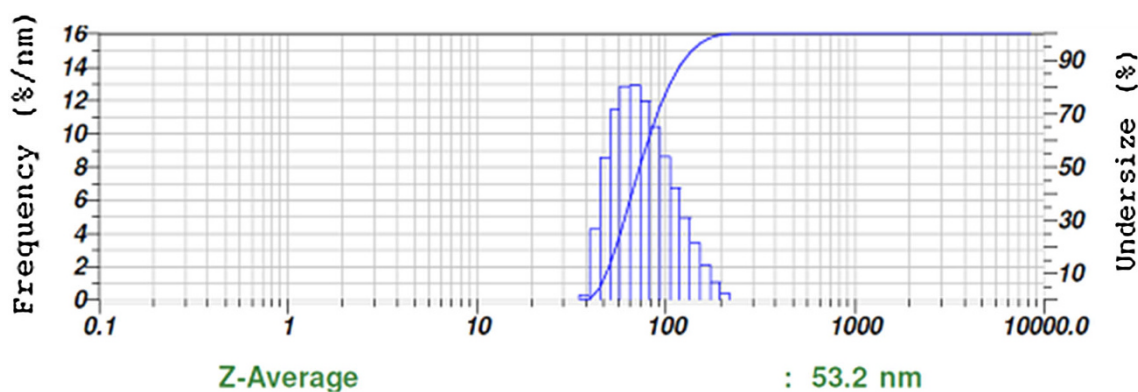


Fig. 5 Particle size distribution curve for AgNPs

Fig. 6 Zeta potential of synthesized AgNPs

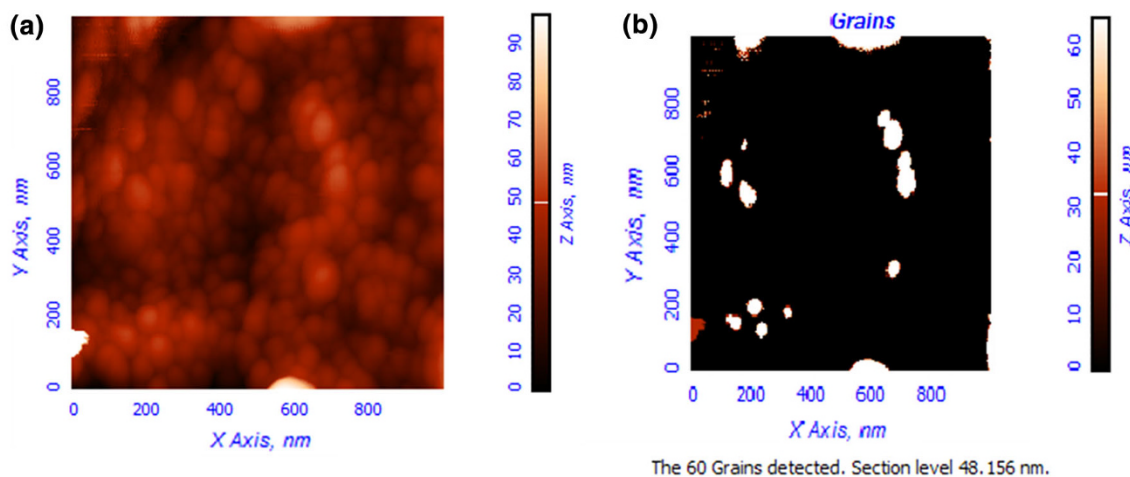
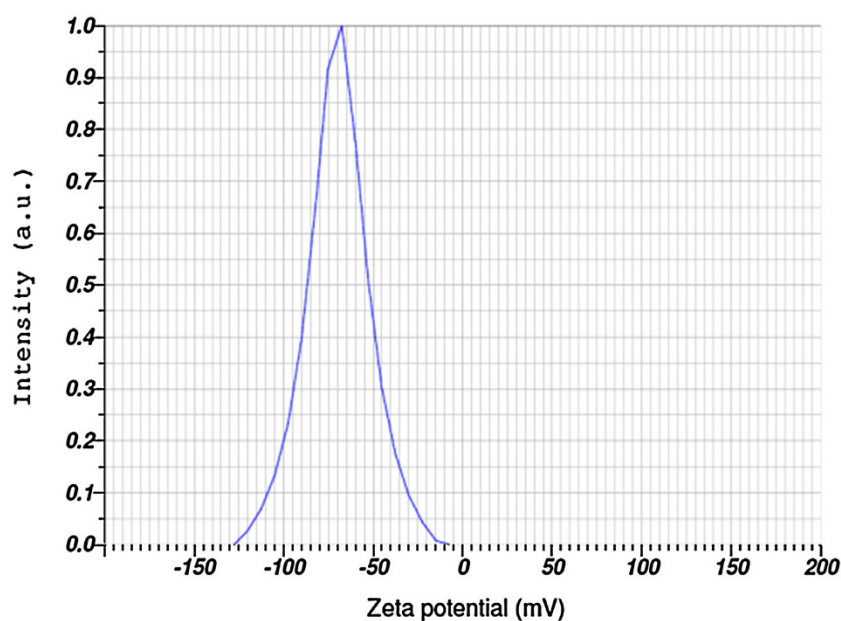


Fig. 7 **a** 2D image of synthesized AgNPs. **b** Grains detected

surface of the aluminum foil. The size of the synthesized AgNPs varied from 40 ± 5 to 85 ± 5 nm. The average grain size of the nanoparticles was detected as 47 ± 3 nm (Fig. 7).

Bactericidal Activity

The nanoparticles synthesized by green route were found very destructive against three bacterial species at a concentration of 30 μ l AgNPs on different strains of antibiotic resistant Gram-negative bacteria *E. coli*. The results revealed superior antibacterial activity against *E. coli* recipient strain and donor strain, where as intermediated activity was revealed against *E. coli* mutant strain. The inhibitory activities in culture media of the Ag nanoparticles reported in (Table 1; Fig. 8) were comparable with reference drug viz. Amoxycylav. Several peoples investigated the antibacterial activity of silver nanoparticles against different bacterial strains (Kotakadi et al. 2013; Rout Rajesh et al. 2009; Kim et al. 2007). In olden days before introduction of penicillin in 1940, silver has been widely used for the treatment of bacterial infection (Hugo and Russell 1982; Chopra 2007). After penicillin invention, the use of silver has been minimized, whereas the use of silver nitrate in the year 1960 by Moyer proved that it possesses very good antibacterial property against *S.*

aureus, *P. aeruginosa*, and *E. coli* (Moyer et al. 1965). There are earlier reports of mechanistic study of inhibition of silver ions against two strains of bacteria, *S. aureus* and *E. coli* (Feng et al. 2000). Other scientists also investigated the effect of silver nanoparticles and compared the results with antibiotics, and reported that the synthesis of silver nanoparticles in the size range of 10–15 nm has very good antibiotic effect on both Gram-negative and Gram-positive microorganisms (Shahverdi et al. 2007). Whereas investigations have also been carried out on antibacterial properties of silver nanoparticles of different shapes and found that the antibacterial efficacy of silver nanoparticles is also shape dependent (Pal et al. 2007). Spherical silver nanoparticles have inhibitory effect at the concentration of 12.5 μ g/ml, whereas truncated triangular nanoparticles have inhibition at 1 μ g/ml of silver content and rod-shaped nanoparticles at the concentration 50–100 μ g/ml content of silver. The studies of silver nanoparticles of spherical-shaped nanoparticles in the size range of 20 nm showed efficient antibacterial activity against *E. coli* and *S. aureus* (Gade et al. 2008).

In the present scenario, silver nanoparticles have made a significant comeback as a potential antimicrobial agent as several pathogenic bacteria have developed resistance against various antibiotics. Silver nanoparticles have emerged up as novel antimicrobial agents due to its high surface area to volume ratio and unique chemical and physical properties (Kim et al. 2007; Morones et al. 2005). Currently, the main interest of many researchers is the development of novel metal ions which have microbial resistance, against the antibiotics resistant strains. Silver nanoparticles (AgNPs) have been proved to be most effective antimicrobial efficacy against bacteria, viruses, and other eukaryotic microorganisms (Gong et al. 2007). Similar type of study was done by (Vanaja and Annadurai 2013), with different type bacteria. But in the present study, we have carried out with antibiotic resistant bacteria.

Once the AgNPs are added to bacterial culture, the silver nanoparticles enter the bacterial cell by diffusion and also by endocytosis through the cell wall into the cytoplasm,

Table 1 Antibacterial activity of the synthesized silver nanoparticles

Bacterial species	Kapparillaku	
	Inhibition zones (mm)	
Concentration	Control (30 mcg)	AgNPs (30 mcg)
<i>E. coli</i> strain I (donor) rifampin resistant	15	18
<i>E. coli</i> strain II (recipient) streptomycin resistant	15	21
<i>E. coli</i> AB 1157 (mutant) streptomycin resistant	17	16

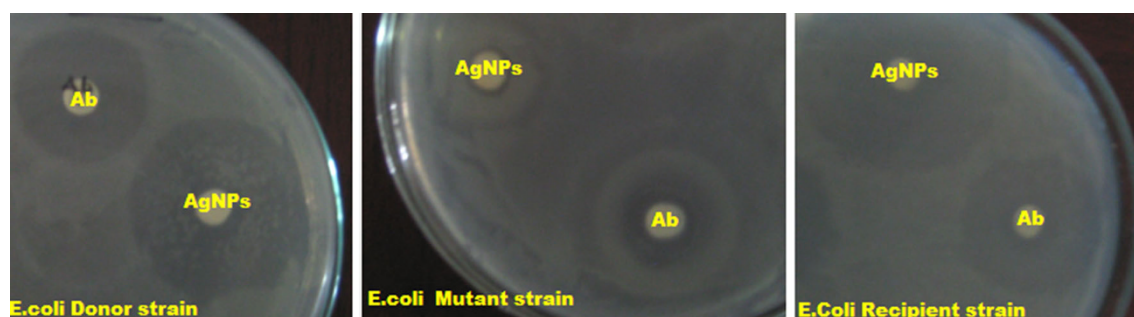


Fig. 8 Bactericidal activity AgNPs against antibiotic resistant strains of *E. coli*

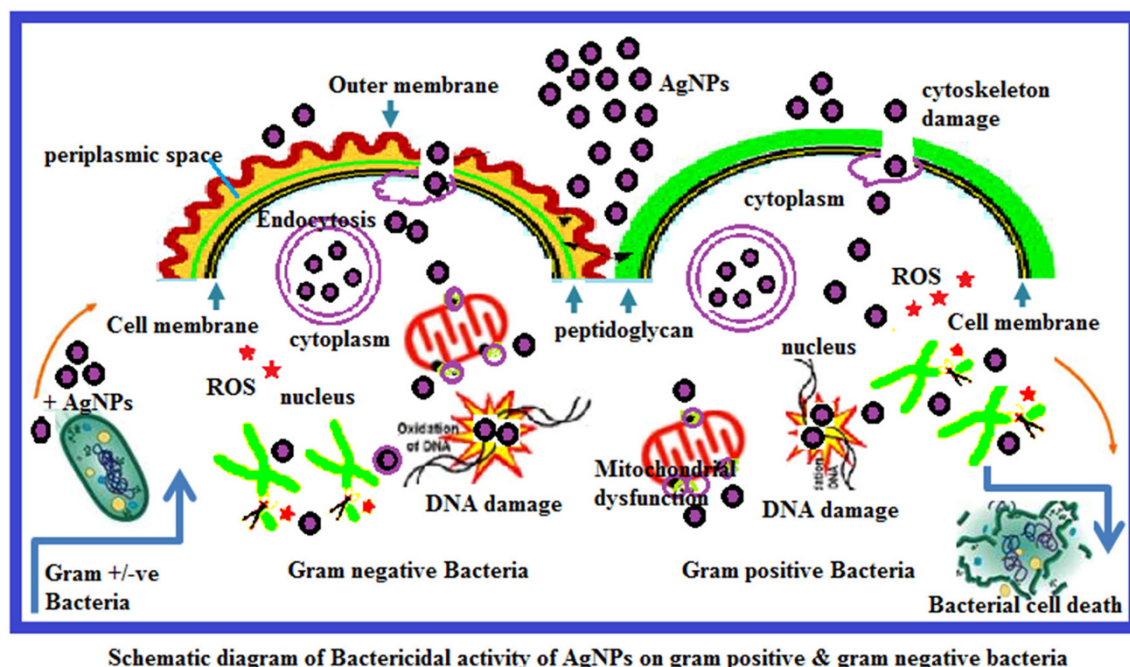


Fig. 9 Schematic diagram of antimicrobial mechanism of AgNPs

and the AgNPs cause toxicity to the bacterial cell constituents, especially the mitochondria is damaged and so the depletion of ATP takes place, which in turn produce reactive oxygen species (ROS). The ROS along with AgNPs acts on nucleus of the bacteria, which cause oxidation of DNA and also cause chromosomal aberrations which ultimately cause the death of the bacteria, so the bacterial cell death occurs. The mechanism is shown in Fig. 9. Schematic diagram. Similar type of results is also presented by scientists (Xiu et al. 2012) on bacteria using different types of silver nanoparticles.

In the present study, the particles have been found to be spherical in size around 45 ± 5 nm, which have excellent bactericidal activity against three different strains of *E. coli* (recipient and mutant strains) which resistance to streptomycin and (donor strain) Rifampin resistant bacteria. The interesting thing is that so far so many scientists have worked on antimicrobial activity using silver and silver nanoparticles on different strains of bacterial species. We are the first to workout on the antibacterial activity against three different antibiotic resistant *E. coli* strains. The results open new avenues for the development of novel antibiotics against multidrug resistant bacteria with eco-friendly and nontoxic green synthesis.

Conclusions

Kapparillaku leaf extract is found to be appropriate for rapid extraction of AgNPs by green synthesis within 5 min.

The average size of silver nanoparticles was found to be 48 ± 5 nm, which have excellent bactericidal activity against three different strains of antibiotic resistant *E. coli*. The nanoparticles <100 nm size are very important in biomedical applications.

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